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# Comparative evaluation of the My5-FU™ immunoassay and LC-MS/MS in monitoring the 5-fluorouracil plasma levels in cancer patients

## Abstract

**Background:** Chemotherapies of solid tumors commonly include 5-fluorouracil (5-FU). With standard doses of 5-FU, substantial inter-patient variability has been observed in exposure levels and treatment response. Recently, improved outcomes in colorectal cancer patients due to pharmacokinetically guided 5-FU dosing were reported. We aimed at establishing a rapid and sensitive method for monitoring 5-FU plasma levels in cancer patients in our routine clinical practice.

**Methods:** Performance of the Saladax My5-FU™ immunoassay was evaluated on the Roche Cobas® Integra 800 analyzer. Subsequently, 5-FU concentrations of 247 clinical plasma samples obtained with this assay were compared to the results obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and other commonly used clinical analyzers (Olympus AU400, Roche Cobas c6000, and Thermo Fisher CDx90).

**Results:** The My5-FU assay was successfully validated on the Cobas Integra 800 analyzer in terms of linearity, precision, accuracy, recovery, interference, sample carryover, and dilution integrity. Method comparison between the Cobas Integra 800 and LC-MS/MS revealed a proportional bias of 7% towards higher values measured with the My5-FU assay. However, when the Cobas Integra 800 was compared to three other clinical analyzers in addition to LC-MS/MS including 50 samples representing the typical clinical range of 5-FU plasma concentrations, only a small proportional bias ( $\leq 1.6\%$ ) and a constant bias below the limit of detection was observed.

**Conclusions:** The My5-FU assay demonstrated robust and highly comparable performance on different analyzers. Therefore, the assay is suitable for monitoring 5-FU plasma levels in routine clinical practice and may contribute to improved efficacy and safety of commonly used 5-FU-based chemotherapies.

**Keywords:** Cobas Integra 800; 5-fluorouracil; method comparison; My5-FU; therapeutic drug monitoring.

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## Introduction

The anticancer drug 5-fluorouracil (5-FU) is commonly used in combination chemotherapies of solid tumors [1]. In general, 5-FU is administered by continuous intravenous infusion, bolus injection or as an oral prodrug formulation (e.g., capecitabine). Substantial inter-individual variability in drug exposure and therapy response has been observed in the Caucasian population at standard 5-FU doses [2, 3]. Approximately 10%–20% of patients treated with 5-FU-based regimens develop severe toxicities of grade 3 or higher as assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE). It has been observed that the majority of patients exhibit drug levels outside the therapeutic range and that more than 50% of patients do not benefit from standard 5-FU therapies [1].

In the current clinical practice, dosing of 5-FU is commonly based on the patient's body surface area (BSA), even though it has been shown that BSA is a poor predictor of systemic drug exposure [4–6]. Several studies have reported a relationship between drug exposure and response in terms of both efficacy and toxicity [5, 7–9]. An area under the curve (AUC) of 20–25 mg×h/L, that is calculated for 5-FU as plasma concentration (mg/L) at steady

state multiplied by infusion time (h), has been established as the target exposure range for optimal treatment outcome in colorectal cancer [10]. By adjusting the 5-FU dosing based on pharmacokinetic monitoring in patients receiving 5-FU monotherapy, significantly improved response rate and fewer gastrointestinal toxicities were observed compared to patients treated with conventional dosing of 5-FU [10]. These findings were recently replicated in a cohort of 157 metastatic colorectal cancer patients receiving either pharmacokinetically guided or conventional 5-FU dosing in FOLFOX (leucovorin, fluorouracil, oxaliplatin) therapy [11]. Therapeutic drug monitoring (TDM) of 5-FU during infusion therapy may be an effective tool to reduce the occurrence of adverse side effects due to drug concentration levels above the optimal range, as well as to identify under-dosed patients receiving less effective treatment. Therefore, 5-FU TDM could improve both safety and efficacy of the treatment.

Routine testing of drug levels requires an analytical method, which is accurate, rapid, and easy to use. Over the last 40 years, a variety of methods for 5-FU analysis have been developed, with recent reports applying liquid chromatography-tandem mass spectrometry (LC-MS/MS) [12–18]. Although, LC-MS/MS methods are very sensitive and robust, the instrumentation is not yet in standard use in routine clinical laboratories. In addition, there is no certified commercial kit assay currently available for measuring 5-FU plasma levels by LC-MS/MS that can be readily used by routine laboratories. However, a nanoparticle immunoassay for 5-FU analysis (My5-FU™; Saladax Biomedical, Inc., PA, USA) was recently developed and evaluated on the Olympus AU400 analyzer (Beckman Coulter, Nyon, Switzerland) [19, 20]. In this study, we evaluated the My5-FU assay performance on the Cobas® Integra 800 analyzer (Roche Diagnostics, Rotkreuz, Switzerland) and compared results from clinical plasma samples to those obtained by LC-MS/MS and by three other clinical analyzers. Our aim was to establish a rapid and sensitive method for monitoring 5-FU plasma levels in cancer patients in our routine clinical practice.

## Materials and methods

### Samples

Clinical samples were collected from 32 gastrointestinal cancer patients receiving prolonged (48 h) 5-FU infusion therapy at the Cantonal Hospital of St. Gallen, Switzerland, according to a study protocol approved by the local Ethics Committee (EKSG 08/088). All

study participants provided written informed consent. A total of 197 samples were taken at several time points during the first two cycles of chemotherapy. Blood samples were collected in heparinized tubes and immediately placed on ice to inhibit dihydropyrimidine dehydrogenase (DPD) enzyme activity to avoid 5-FU degradation [21]. Plasma was separated within 1 h after blood collection and samples were stored at  $-20^{\circ}\text{C}$  until analysis. Plasma samples from healthy volunteers were obtained using the same procedure as described above, and if needed, pooled and spiked to assess different validation parameters. In addition, a set of 50 samples (banked samples from patients on 5-FU-containing regimens) was provided by Saladax Biomedical for method comparison.

### My5-FU assay

The My5-FU immunoassay was developed for rapid determination of 5-FU levels in human plasma [19]. The assay is based on aggregation of nanoparticles that is inversely proportional to the amount of 5-FU in the sample, and it can be applied to automated clinical chemistry analyzers with photometric detection. The assay reagents, calibrators, and quality control (QC) samples for the study were provided by Saladax Biomedical. The calibrator set contained standards at 0, 150, 300, 600, 1200, and 1800 ng/mL of 5-FU, and the three QC samples (low, medium, and high control) contained 5-FU at concentrations of 225 ng/mL, 450 ng/mL, and 900 ng/mL, respectively.

### Assay validation on Cobas Integra 800

The performance of the My5-FU assay was evaluated on the Cobas Integra 800 analyzer. The following validation parameters were assessed: linearity, within-run precision, within-run accuracy, total precision, between-day precision, between-day accuracy, recovery, interference, sample carryover, and dilution integrity. Prior to each run, a calibration and duplicate QC analyses were performed. QC samples had to be within the specifications recommended by the manufacturer:  $\pm 8\%$  for the low QC sample (range 207–243 ng/mL),  $\pm 6\%$  for the medium QC sample (range 423–477 ng/mL), and  $\pm 5\%$  for the high QC sample (range 855–945 ng/mL).

Linearity was assessed using five replicates of 11 different 5-FU concentrations covering the calibration range. Samples for linearity assessment were obtained by diluting plasma spiked at a high concentration of 5-FU (1750 ng/mL) with plasma spiked at a low concentration (100 ng/mL) as described in the Clinical Laboratory Standards Institute (CLSI) Guideline EP6-A [22]. Linearity was considered acceptable if the imprecision (coefficient of variation, CV) at each concentration level was  $\leq 15\%$  and the inaccuracy (percent bias, %bias) was within  $\pm 10\%$  for concentrations  $\geq 150$  ng/mL and within  $\pm 15\%$  for concentrations  $< 150$  ng/mL.

Within-run imprecision (CV) was determined on two separate days by analyzing on each day 20 replicates of the low and the medium QC samples and two plasma pools (pooled from five healthy donors), which were spiked with 5-FU at concentrations of 240 and 700 ng/mL. Imprecision of  $\leq 10\%$  was considered acceptable following the manufacturer's recommendation. No specifications for within-run inaccuracies were stated by the manufacturer. However, according to internationally accepted guidelines for bioanalytical method validation [23, 24], within-run inaccuracies (%bias) should

not exceed 15%. The total imprecision (CV) was determined by analyzing 40 replicates of each QC sample in five days (two replicates/sample; two samples/run; two runs/day) with a CV specification limit of  $\leq 15\%$  stated by the manufacturer. Additionally, a between-day imprecision (CV) over 31 days was evaluated by analyzing one replicate of each QC sample per day. If results of the QC replicates were outside the specification range, the samples were re-analyzed and both replicates included in the between-day imprecision calculation. All three QC concentration levels over these 31 days had to be re-analyzed 14 times, thus, the between-day imprecision is reported with a total of 45 replicates per QC sample. Between-day CV of  $\leq 15\%$  was considered acceptable following the manufacturer's recommendation. Similarly, a between-day inaccuracy (%bias) was evaluated applying a specification limit of  $\pm 15\%$  [23, 24].

Percentage recovery was assessed in 5-FU-free lithium-heparin plasma spiked at the following 5-FU concentrations: 250, 500, 1000, 1250, and 1600 ng/mL. Five replicates per sample were analyzed.

The percentage interference for compounds potentially present in plasma, i.e., lipids, bilirubin, protein, and rheumatoid factor, was assessed using the following procedure: Samples for intra-lipid (mean concentration of 1864 mg/dL), protein (human immunoglobulin G; 12.5 g/dL), and rheumatoid factor (500 units/dL) testing were prepared by adding the respective interferent or an appropriate control (i.e., saline or plasma) to a pooled plasma sample, which was spiked with 1200 ng/mL 5-FU, following the CLSI Guideline EP7-A2 [25]. Accordingly, five anonymous routine samples with a mean bilirubin concentration of 100  $\mu\text{mol/L}$  were spiked with 1000 ng/mL 5-FU to assess the relative recovery of 5-FU compared to a control sample in presence of bilirubin. For interference, a specification limit of  $\pm 10\%$  was defined by the manufacturer.

Sample carryover was assessed in one run with randomly distributed replicates of two calibrator samples with low ( $n=11$ ) or high ( $n=10$ ) 5-FU concentration, 150 or 1800 ng/mL, respectively. Finally, the dilution integrity of plasma samples was evaluated up to 10-fold using water, zero calibrator standard, and 0.9% sodium chloride solution (B. Braun, Melsungen, Germany).

## Method comparison

For method comparison, 247 human plasma samples were analyzed with the My5-FU immunoassay and an LC-MS/MS assay. Of these, a set of 50 samples was provided by Saladax Biomedical, whereas the rest of the samples were from cancer patients treated with 5-FU-based regimens at the Cantonal Hospital of St. Gallen. Samples containing 5-FU concentrations above the linear calibration range of the My5-FU assay were diluted up to 10-fold with water before analysis. Most of the LC-MS/MS analyses were performed as described earlier on a QTRAP® 4000 linear ion trap quadrupole mass spectrometer (AB SCIEX, Darmstadt, Germany) [12]. A subset of samples was analyzed applying the same method with fine adjustments to a newer version of this LC-MS/MS system, i.e., a QTRAP 5500 (AB SCIEX). The specific MS/MS parameters can be requested from the authors. The results obtained on Cobas Integra 800 analyzer and LC-MS/MS for the subset of 50 samples were also compared to results obtained using the My5-FU assay on three other automated clinical analyzers, namely Olympus AU400, Roche Cobas c6000, and Thermo Fisher CDx90 (data provided by Saladax Biomedical).

For each method comparison (method 1 vs. method 2), results were compared using the Passing-Bablok regression analysis generating a linear equation with calculated values for intercept and slope [26]. The intercept value represents a measure for potential systematic differences between methods. If the 95% confidence interval (CI) of the intercept contains zero, methods show no evidence for a systematic difference. However, if the 95% CI does not include zero, a constant bias between the methods is present. Accordingly, if the 95% CI of the slope does not include one, a proportional bias between the methods is detected.

To test the validity of the linear model, the Passing-Bablok analysis includes a cumulative sum (Cusum) linearity test [26]. The threshold for rejecting the null hypothesis of a linear relationship was set to  $\alpha=10\%$ . Correlation analysis was additionally performed for all method comparisons obtaining a squared Pearson correlation coefficient ( $R^2_{\text{Pearson}}$ ).

The degree of agreement between methods was further evaluated by using the Bland-Altman analysis, plotting the mean percent difference against the average of both methods [27].

The method comparison analyses were performed with the Analyse-it® v2.20 software add in for Microsoft® Excel™ (Microsoft Corporation, Redmond, WA, USA).

## Results

### Assay validation on Cobas Integra 800

The assay was found to be linear over the entire concentration range of 5-FU (100–1750 ng/mL). Linearity was described with the following linear regression equation:  $y=1.09 \times x - 25.9$ . The CV of all concentration levels was  $\leq 3.7\%$ , and the %bias was  $\leq 7.4\%$ .

The defined specification criteria were met for imprecision and inaccuracy. Within-run imprecision was tested by analyzing 20 replicates of the low and the medium QC sample and two pooled plasma samples spiked with 5-FU on two separate days. For QC samples, the within-run CV ranged from 0.95% to 3.9% and for pooled plasma samples from 0.65% to 2.2% (Table 1), being within the acceptable specification limit ( $\leq 10\%$ ). The within-run %bias for QC samples ranged from 4.8% to 7.4% and for pooled samples from 2.2% to 8.6%, being also within the specification criteria ( $\pm 15\%$ ). Total CV ( $n=40$ ) for the low, the medium, and the high QC concentration levels were 3.5%, 2.7%, and 1.4% (specification limit:  $\leq 15\%$ ), respectively. Similarly, between-day CVs ( $n=45$ ) of 5.6%, 3.5%, and 2.1% (specification limit:  $\leq 15\%$ ) and %biases of 3.7%, 3.8%, and 2.4% (specification limit:  $\pm 15\%$ ) were obtained for the respective QC samples.

The percentage recovery for different 5-FU concentrations ranged from 99% to 105% (Table 2), and was thus within the acceptable range (96%–108%) given by the manufacturer.

**Table 1** Within-run imprecision and inaccuracy of quality control and two plasma pool samples spiked with 5-FU.

Sample type	Replicates	Nominal 5-FU concentration, ng/mL	Mean 5-FU concentration, ng/mL	Standard deviation (SD)	Imprecision (CV), %	Inaccuracy (%bias)
QC, low						
Day 1	20	225	236	8.62	3.7	4.8
Day 2	20	225	239	9.33	3.9	6.2
Day 1 and 2	40	225	237	9.01	3.8	5.5
QC, medium						
Day 1	20	450	479	7.78	1.6	6.5
Day 2	20	450	483	4.57	0.95	7.4
Day 1 and 2	40	450	481	6.68	1.4	6.9
Plasma pool 1						
Day 1	20	240	255	5.65	2.2	6.2
Day 2	20	240	261	3.57	1.4	8.6
Day 1 and 2	40	240	258	5.51	2.1	7.4
Plasma pool 2						
Day 1	20	700	715	6.41	0.90	2.2
Day 2	20	700	734	4.79	0.65	4.9
Day 1 and 2	40	700	725	11.1	1.5	3.5

CV, coefficient of variation; QC, quality control.

**Table 2** My5-FU assay recovery of 5-FU in lithium-heparin plasma at five different concentrations (n=5).

Parameters	1	2	3	4	5
Nominal 5-FU concentration, ng/mL	250	500	1000	1250	1600
Mean 5-FU concentration, ng/mL	252	507	992	1309	1597
Standard deviation (SD)	3.0	3.8	6.1	11	18
Recovery, %	101	101	99.2	105	99.8
Imprecision (CV), %	1.2	0.76	0.62	0.83	1.1
Inaccuracy (%bias)	0.80	1.3	-0.84	4.8	-0.21

CV, coefficient of variation.

Low interference by bilirubin (−5%), total protein (4%), and rheumatoid factor (−2%) was observed. However, intra-lipids showed an interference of 11%.

Sample carryover was evaluated using randomly distributed replicates (n=21) of low and high 5-FU calibrator samples (150 and 1800 ng/mL, respectively). Since a CV of 2.6% and %bias of 7.4% was observed for the low concentration sample, the preceding high concentration sample had no influence on the low sample indicating the absence of any carryover effects.

Finally, a 10-fold dilution of three clinical plasma samples with water, zero calibrator standard or 0.9% sodium chloride solution yielded an imprecision of ≤10%.

## Method comparison

Of the 247 clinical samples analyzed for the method comparison, 107 (43%) contained 5-FU concentrations above

the linear calibration range (>1800 ng/mL) of the My5-FU assay requiring up to 10-fold dilution. The 5-FU concentrations determined by LC-MS/MS showed a range from 102 to 18590 ng/mL with a median concentration of 1270 ng/mL. Similarly, the 5-FU concentrations obtained with the My5-FU assay ranged from 93 to 17881 ng/mL with a median concentration of 1342 ng/mL.

All method comparison statistics are shown in Table 3. The comparison between the My5-FU assay on the Cobas Integra 800 analyzer and the LC-MS/MS method yielded a  $R^2_{\text{Pearson}}$  of 0.987. The Passing-Bablok analysis of this comparison is shown in Figure 1. A linear equation of Cobas Integra 800 (ng/mL)=1.08×LC-MS/MS (ng/mL)−3.96 (95% CI slope 1.06–1.09; 95% CI intercept −14.5–11.4) was obtained (Table 3). The CI of the slope indicated a proportional bias of 8% higher values for the Cobas Integra 800 as compared to the LC-MS/MS method. Accordingly, the Bland-Altman method revealed a similar proportional bias of 7% between the two methods (Figure 2).

Method comparison estimates between the other clinical analyzers and the Cobas Integra 800 are listed in Table 3. The performance of the My5-FU assay was comparable between the different instruments all showing a  $R^2_{\text{Pearson}}$  of ≥0.993. They all, however, revealed a similar proportional bias with slightly higher values being obtained with the Cobas Integra 800 analyzer. In addition, a constant bias below the limit of detection (<52 ng/mL) was observed between Cobas Integra 800 and all other clinical analyzers.



**Table 3** Method comparison statistics.

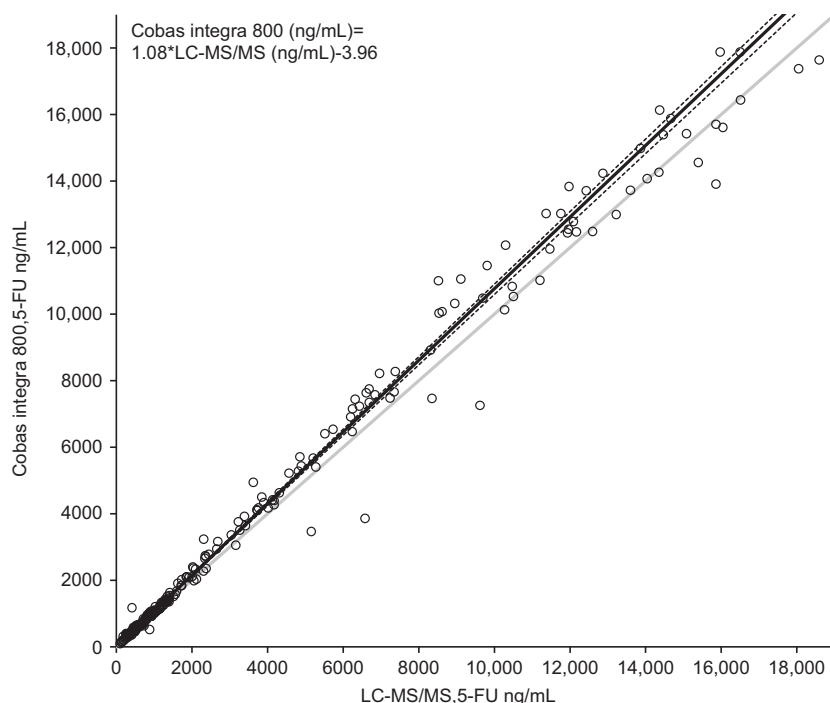
Method 1	Method 2	n	Concentration range of method 1, ng/mL	Slope <sup>a</sup> (95% CI)	Intercept <sup>a</sup> (95% CI)	Cusum p-value <sup>b</sup>	%bias <sup>c</sup> (95% CI)	$R^2_{\text{Pearson}}$
LC-MS/MS	Integra 800	247	102–18 590	1.08 (1.06–1.09)	–3.96 (–14.5–11.4)	>0.1	7.0 (5.5–8.5)	0.987
Integra 800	LC-MS/MS	50	93–1581	0.97 (0.95–0.99)	12.0 (–2.20–19.0)	>0.1	–1.4 (–2.6–0.2)	0.996
Integra 800	AU400	50	93–1581	0.96 (0.95–0.97)	18.1 (12.6–26.5)	>0.1	0.2 (–1.5–1.9)	0.999
Integra 800	CD×90	50	93–1581	0.94 (0.93–0.95)	32.3 (23.4–43.5)	>0.1	0.6 (–1.1–2.3)	0.998
Integra 800	c6000	45	93–1581	0.96 (0.95–0.99)	19.8 (9.90–32.2)	>0.1	1.6 (0.1–3.1)	0.993
LC-MS/MS	Integra 800	50	102–1560	1.03 (1.01–1.05)	–12.3 (–19.9–2.22)	>0.1	1.4 (0.2–2.6)	0.996
LC-MS/MS	AU400	50	102–1560	0.99 (0.97–1.01)	11.2 (–2.75–26.7)	>0.1	1.6 (–0.1–3.2)	0.995
LC-MS/MS	CD×90	50	102–1560	0.97 (0.95–0.99)	21.6 (6.95–38.9)	>0.1	2.0 (0.3–3.6)	0.992
LC-MS/MS	c6000	45	102–1560	0.99 (0.97–1.02)	10.8 (–3.63–33.7)	>0.1	2.7 (1.1–4.2)	0.991

<sup>a</sup> Passing-Bablok regression parameter; <sup>b</sup> Cusum linearity test of Passing-Bablok fit; <sup>c</sup> Percentage of the mean difference according to Bland Altman. CI, confidence interval.

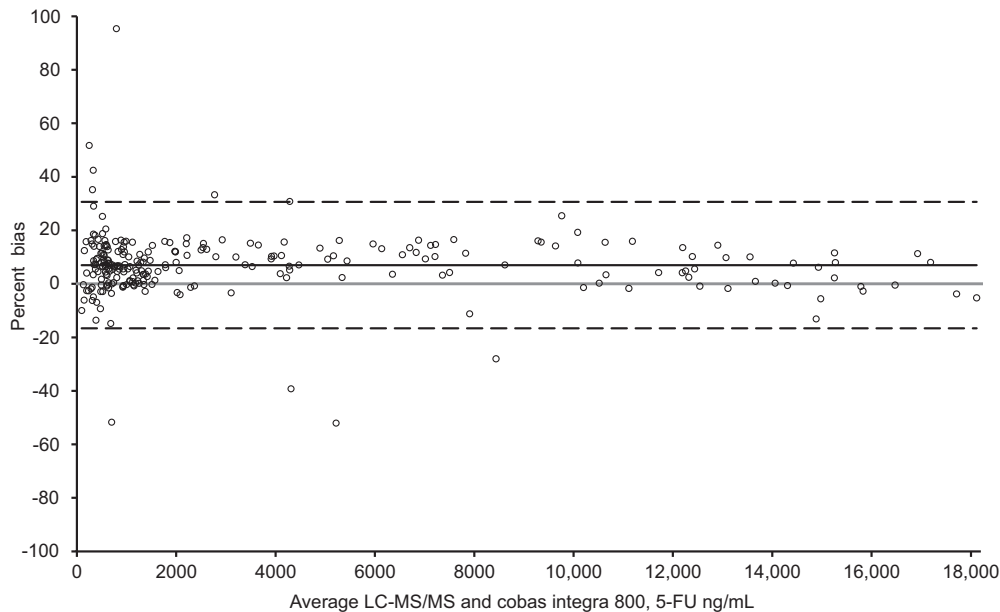
## Discussion

The My5-FU assay was successfully established on the Cobas Integra 800 analyzer and results from the analysis of 247 clinical samples showed good correlation with LC-MS/MS. Compared to the previously published validation data on Olympus AU400 analyzer [20], we report very similar imprecision characteristics: within-run CV of 3.9% vs. 2%, and a total CV of 3.5% vs. 3.9%. Overall, the assay validation criteria were met for linearity, precision, accuracy, recovery, sample carryover, and dilution integrity.

However, an interference of 11% was observed for intralipids. This could be explained by a freezing effect, since the interference was gradually increasing while the sample was re-frozen and thawed between the analyses. Although the assay has been validated for three freeze-thaw cycles, it is recommended to keep plasma samples at 4 °C until analysis, and freeze sample aliquots for longer storage. Furthermore, the preanalytical handling of the samples may have a great influence on the quality of the results. Most importantly, the residual DPD enzyme activity in the sample needs to be inhibited immediately after

**Figure 1** Passing-Bablok analysis of the Cobas Integra 800 compared to LC-MS/MS (n=247).

The black full line is the regression line and the two dashed lines show the 95% CI of its slope. The grey full line is the identity line (slope=1).



**Figure 2** Bland-Altman plot of the Cobas Integra 800 compared to LC-MS/MS.

The grey full line represents the identity line (i.e., full agreement between both methods), whereas the black full line indicates the bias between the methods. The two dashed lines show the 95% CI.

blood drawing as it will lead to artificially lowered 5-FU plasma concentrations, and thus, to the risk of missing patients at high risk of toxicity due to elevated steady-state 5-FU levels. The inhibition of the residual enzyme activity may be achieved by immediate cooling of the sample on ice before plasma separation, or by adding a commercially available stabilizer (provided by Saladax Biomedical) to the sample.

The Passing-Bablok and Bland-Altman analyses revealed a proportional bias towards higher values using the My5-FU assay on the Cobas Integra 800 analyzer compared to LC-MS/MS. Accordingly, slightly higher concentrations of 5-FU compared to the nominal concentrations were generally observed for QC and spiked plasma samples using the My5-FU assay (Table 1). However, the precision and the accuracy were within the acceptable range and a positive bias of 7% would not lead to an unintentional under-dosing of a patient who is on the optimal AUC target level ( $22.5 \text{ mg} \times \text{h/L} \pm 11.1\%$ ). The apparent lack of such a proportional bias in the comparisons between the My5-FU assay on other clinical chemistry analyzers and the LC-MS/MS assay may be largely explained by the more than 10-fold extended range of concentration values and the five-fold larger sample size that were included in the comparison of the Cobas Integra 800 and LC-MS/MS (93–18590 ng/mL vs. 93–1581 ng/mL; Table 3). Indeed, the proportional bias in the comparison of the Cobas Integra 800 analyzer and LC-MS/MS is strongly reduced to 1.4% if the same smaller set of samples is compared (Table 3).

Since the standard infusional regimens, such as FOLFOX, use an infusion time of 48 h, the optimal steady-state levels are low (i.e., 417–521 ng/mL with the target AUC of 20–25  $\text{mg} \times \text{h/L}$ ). Thus, the data included in the additional method comparison series based on a subset of samples ( $n=50$ ) analyzed by using the Cobas Integra 800 and three other clinical chemistry analyzers in addition to LC-MS/MS, were within a clinical range of plasma concentrations. Our results suggest that the performance of the My5-FU assay on the different analyzers is highly comparable (Table 3). Compared to the LC-MS/MS assay, only a small proportional bias and a constant bias below the limit of detection ( $<52 \text{ ng/mL}$ ) were observed.

In conclusion, we have demonstrated that the evaluated method is reliable for routine monitoring of 5-FU plasma levels in cancer patients. We confirmed the assay robustness on different analyzers commonly used in clinical laboratories. Steady-state plasma concentration levels of 5-FU are rapidly provided for physicians and the patient's treatment can be adjusted if necessary. This is possible because standard 5-FU therapy regimen cycles are biweekly, and delayed toxicity symptoms may occur up to several days or weeks after the first cycle of therapy. Monitoring 5-FU plasma levels may provide an effective approach to improve efficacy and safety of commonly used 5-FU-based therapies in cancer patients.

**Acknowledgments:** The My5-FU assay reagents, calibrators, pooled plasma samples for validation, 50 clinical

plasma samples, and data for a subset of samples (AU400, CDx90, Cobas c6000) for method comparison were kindly provided by Saladax Biomedical. We thank J. Dias and U. Sonnenschein for their support in the My5-FU assay evaluation and P. Rhyn and C. Bühr for their expertise and help with the LC-MS/MS analyses.

### Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding

the publication of this article. Research support played no role in the analysis and interpretation of data, in the writing of the report or in the decision to submit the report for publication.

**Research funding:** Financial support for this study was provided by a research grant from the Swiss National Science Foundation (31003A\_138285) to Carlo R. Largiadèr.

**Employment or leadership:** None declared.

**Honorarium:** None declared.

Received September 21, 2012; accepted January 21, 2013

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